Remarks

Upon entry of the foregoing amendment, claims 1-10 are pending in the application, with claims 1 and 7 being the independent claims. The specification has been amended only to direct the entry of Substitute Sequence Listing and provide the sequence identification numbers next to the specific sequence. Claims 1, 3 and 5-10 have been amended to correct typo and grammar errors, to eliminate multiple dependent claims and to insert sequence identification numbers next to the specific sequence. Thus, no new matter has been added by the amendment and entry and consideration of the same is respectfully requested.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Substitute Sequence Listing and the computer readable copy of the Substitute Sequence Listing submitted herewith in the above-captioned application are the same.

Conclusion

It is respectfully believed that the present application is in condition for examination. Early notice to this effect is earnestly solicited. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Respectfully submitted,

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Version With Markings to Show Changes Made

In the Specification:

The specification has been amended as follows:

At page 5, the paragraph starting at line 20 and ending at line 22 was replaced with the following paragraph:

> Peptide inhibitors developed on the basis of the cleavage recognition site of human SCC1, "DREIMR" (SEQ ID NO:9), were also able to inhibit separase at a similar concentration as the above mentioned peptide inhibitors (Fig. 3).

At page 5, the paragraph starting at line 24 and ending at line 31was replaced with the following paragraph:

> It was further tested which form of separase represents the active protease. It was found that the peptide inhibitor Bio-SVEQGR(SEQ ID NO:10)-amk bound exclusively to the cleaved forms of human separase when the peptide derivative was added to separase after its activation in mitotic Xenopus extracts. This observation shows that the active site of separase is accessible to Bio-SVEQGR(SEQ ID NO:10)-amk in the cleaved, but not in the residual amount of full-length separase to which securin is still bound (Fig. 4). These results suggest that the cleaved forms of separase represent active forms of the protease.

At page 9, the paragraph starting at line 10 and ending at line 30 was replaced with the following paragraph:

> Once the active form(s) of human separase have been obtained by one or more of the methods described above, synthetic peptide substrates for separase are designed and synthesized that allow the simple detection of protease activity in high throughput format, e.g. by fluorogenic methods. The proteolytic assays suitable for this purpose have been described in WO00/48627. By way of example,

substrate peptides containing the separase recognition sequence (see WO00/48627) that carry a C-terminal fluorophore such as a 7-amino-4-methyl-coumarin group (AMC) are synthesized by standard methods. The cleavage of AMC (or other fluorophore groups used) results in a rise in fluorescence which can be measured fluorometrically. In an experiment of the present invention, the activity of mitotically activated immunoprecipitates of separase (Waizenegger et al., 2000) was measured fluorometrically by using AMC-coupled peptides based on the cleavage recognition sites of human separase, "SFEILR" (SEQ ID NO:11). The result of this experiment provides the basis for the development of a screening assay for identifying separase inhibitors. For conducting this assay in the high throughput mode, compounds, e.g. from chemical or natural product libraries, can be tested for their ability to inhibit the cleavage of fluorogenic peptide substrates by the active form(s) of human separase, which is preferably employed in the screen in recombinant form.

At page 12, the paragraph starting at line 31 and ending at page 13 line 6 was replaced with the following paragraph:

As an alternative to identifying small molecules in a screening method, separase inhibitors can be obtained starting from the recombinant active separase. In this approach, synthetic peptide derivatives, exemplified by derivates of SVEQGR (SEQ ID NO:10), DREIMR (SEQ ID NO:9), SFEILR (SEQ ID NO:11) or EWELLR (SEQ ID NO:12) (e.g. Bio-SVEQGR(SEQ ID NO:10)-amk) can be used as the structural basis to develop peptidomimetic molecules that inhibit separase. For inhibitors of human separase, the cleavage sequence of human SCC1 or human separase can preferably be used. The assays described above using recombinant active separase and peptide substrates, e.g. fluorogenic peptides, can be used to optimize such compounds.

At page 17, the paragraph starting at line 26 and ending at page 18 line 2 was replaced with the following paragraph:

A. Mitotically activated separase immunoprecipitates were preincubated with yeast peptides (Uhlmann et al., 2000) before they were used in the SCC1-cleavage assay. Biotin-

SVEQGR(SEQ ID NO:10)-amk or Biotin-SVEQGR(SEQ ID NO:10)-cmk were used at different concentration (0.1, 1, 10, 100, 1000 M in XB + 0.5 mM DTT). Separase immunoprecipitates were incubated for 10 minutes, 22° C and 1200 rpm. The SCC1 in vitro cleavage assay was performed after washing once with XB + 0.5 mM DTT (see above). Human peptides, Biotin-DREIMR(SEQ ID NO:9)-amk or DREIMR(SEQ ID NO:9)-amk, were diluted and used as above described for the yeast peptides.

At page 18, the paragraph starting at line 4 and ending at line 7 was replaced with the following paragraph:

B. Separase immunoprecipitates were incubated with 100 M Biotin-SVEQGR(SEQ ID NO:10)-amk or with DMSO prior mitotic activation. Incubation for 10 minutes, 22° C and 1000 rpm. After washing twice in XB + 1mM DTT the activation was performed in mitotic Xenopus egg extracts.

At page 18, the paragraph starting at line 9 and ending at line 11 was replaced with the following paragraph:

Interphase Xenopus egg extracts were driven into mitosis in the presence of 1 mM Biotin-SVEQGR(SEQ ID NO:10)-amk or DMSO. Those mitotic Xenopus egg extracts were used to activate a batch of separase immunoprecipitates.

At page 21, the paragraph starting at line 7 and ending at line 26 was replaced with the following paragraph:

To further study the mechanism of separase activation it was tested if two different peptide inhibitors developed to inhibit separase from budding yeast (Uhlmann et al., 2000) are able to inhibit the protease activity of human separase. These inhibitors are synthetic peptides containing the cleavage site of budding yeast SCC1, "SVEQGR" (SEQ ID NO:10), where the last arginine residue represents the P1 site after which separase cleaves. The C-terminus of this peptide is either modified to a chloromethyl ketone (cmk) or to an acyloxymethyl ketone (amk). Both peptide derivatives are

coupled to biotin moieties at their N-termini. The two inhibitors are therefore called Bio-SVEQGR(SEQ ID NO:10)-cmk and Bio-SVEQGR(SEQ ID NO:10)-amk. When these inhibitors were added to human separase that had been isolated by immunoprecipitation and had been activated in mitotic Xenopus extracts as above, it was observed that both inhibitors are able to block the ability of separase to cleave SCC1 (Figure 2A). The concentration of these peptide derivatives required to inhibit human separase was similar to the concentration needed to inhibit separase from budding yeast (compare Fig. 2, upper panel and WO 00/48627, Uhlmann et al., 2000). It was further observed in this experiment that the formation of the p55 cleavage product of human separase was largely inhibited by both peptides at the same concentration at which the ability of separase to cleave SCC1 was inhibited (Figure 2B).

At page 21, the paragraph starting at line 30 and ending at page 22 line 4 was replaced with the following paragraph:

Fig. 2B: Separase immunoprecipitates obtained from nocodazole arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were activated in mitotic Xenopus egg extracts. Subsequently samples were incubated with indicated concentrations of yeast peptides (Biotin-SVEQGR(SEQ ID NO:10)-cmk or Biotin-SVEQGR(SEQ ID NO:10)–amk). After a short wash samples were mixed with SCC1-myc reaction mix for 1 hour. Samples were analysed by immunoblotting with antibodies against myc. Cleaved SCC1 is marked by an arrow.

At page 22, the paragraph starting at line 14 and ending at line 26 was replaced with the following paragraph:

Separase immunoprecipitates obtained from nocodazole-arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were activted in mitotic Xenopus egg extracts. Subsequently samples were incubated with the indicated concentrations of human peptides (DREIMR(SEQ ID NO:9)-amk or Biotin-DREIMR(SEQ ID NO:9)-amk). After washing samples were mixed with SCC1-myc reaction mix for 1 hour. Samples were analysed by immunoblotting with antibodies against

myc. For control a sample was treated with the same concentration of DMSO which was used for the solubilization of the peptides (DMSO). The SCC1-myc reaction mix was loaded as an input control (SCC1-myc input). It was found that peptide inhibitors developed on the basis of the cleavage recognition site of human SCC1, "DREIMR" (SEQ ID NO:9), were able to inhibit separase at a similar concentration as the peptide inhibitors derived from yeast (Fig. 3).

At page 23, the paragraph starting at line 6 and ending at line 22 was replaced with the following paragraph:

To further test which form of separase represents the active protease, the ability of Bio-SVEQGR(SEQ ID NO:10)-amk, the more effective one of the two yeast peptide inhibitors, to bind to different forms of separase, was tested. Previous work has shown that the peptide derivatives inhibit separase by covalently binding to an active site cysteine residue within separase (Uhlmann et al., 2000). These binding reactions can be directly visualized by separating the separase-inhibitor conjugate by SDS gel electrophoresis and by subsequently labeling the biotin moiety on the peptide derivative by streptavidin (Uhlmann et al., 2000). By using this method it was found that Bio-SVEQGR(SEQ ID NO:10)-amk bound exclusively to the cleaved forms of human separase when the peptide derivative was added to separase after its activation in mitotic Xenopus extracts (Figure 4B, lane 3i). This treatment inhibited SCC1 cleavage by separase (Figure 4C, lane 3i). This observation shows that the active site of separase is accessible to Bio-SVEQGR(SEQ ID NO:10)-amk in the cleaved, but not in the residual amount of full-length separase, confirming the conclusion from the securin addback experiments that the cleaved forms of separase represent active forms of the protease.

At page 23, the paragraph starting at line 24 and ending at page 24 line 2 was replaced with the following paragraph:

When Bio-SVEQGR(SEQ ID NO:10)-amk was added to human separase immunoprecipitates before separase had been activated in mitotic Xenopus extracts, only the small amount of p60 that is already present in these

immunoprecipitates was labeled by the peptides, whereas full-length separase was not (Figure 4B, lane 1i), further confirming that the active site of separase is only accessible in the cleaved forms. When the peptide inhibitor was washed away before the separase was subsequently incubated in mitotic Xenopus extracts separase could be activated normally to cleave SCC1 (Figure 4C, lane 1i). This result suggests that the presence of securin prevents the binding of Bio-SVEQGR(SEQ ID NO:10)-amk to the active site of separase, implying that securin inhibits separase by directly or indirectly blocking the access of substrates to the active site of separase.

At page 24, the paragraph starting at line 4 and ending at line 16 was replaced with the following paragraph:

When the activation of human separase immunoprecipitates in mitotic Xenopus extracts was carried out in the presence of Bio-SVEQGR(SEQ ID NO:10)-amk both the cleaved forms of separase and full-length separase were covalently labeled with the peptide (Figure 4B, lane 2i) and separase was unable to cleave SCC1 (Figure 4C, lane 2i). This observation suggests that the full-length form of separase is also transiently active, presumably once its bound inhibitor securin has been destroyed, but that this form is normally labile because it is further processed into the cleaved forms by autocleavage. It was further shown that binding of recombinant securin to cleaved active separase prevented the binding of peptide inhibitors to the active site of separase (Fig. 4D). This result suggests that securin inhibits separase by either directly or indirectly blocking the access of substrates to the active site of separase.

At page 24, the paragraph starting at line 24 and ending at page 25 line 2 was replaced with the following paragraph:

Fig. 4B: Separase IPs (see A) were either preincubated with Biotin-SVEQGR(SEQ ID NO:10)-amk (preinc. with inh. peptide) or with DMSO (preinc. with DMSO), subsequently washed and aliquots were taken. Thereafter they were incubated in mitotic Xenopus egg extracts and washed again. Samples were taken for analysis (1i, 1c). Separase IPs were

activated either in mitotic Xenopus egg extracts which were driven into mitosis in the presence of Biotin-SVEQGR(SEQ ID NO:10)-amk or DMSO, subsequently washed and aliquots were taken for analysis (2i, 2c). Already mitotically activated separase IPs (see A) were incubated with Biotin-SVEQGR(SEQ ID NO:10)-amk or DMSO, thereafter washed and aliquots were taken for analysis (3i, 3c). All samples were analysed by immunoblotting with avidin.

At page 29, the paragraph starting at line 5 and ending at line 22 was replaced with the following paragraph:

The cleavage sites in human separase were mapped by a method that has been previously used to map cleavage sites in SCC1 (WO00/48627). Briefly, truncated versions of the human separase cDNA were generated by polymerase chain reactions, and the resulting cDNAs were used directly for coupled in vitro transcription-translation reactions by using rabbit reticulocyte lysates. The transcription-translation reactions were carried out in the presence of ^{35}S-labeled methionine and cysteine, resulting in radiolabeled translation products. These were then separated by SDS gel electrophoresis side by side with the in vitro cleavage products of mitotically activated human separase immunoprecipitates which were detected by immunoblotting. The comparison of the electrophoretic mobility of a series of deletion mutants with the mobility of the in vitro cleavage products narrows down the regions of cleavage to about 10 amino acid residues. Because separase cleavage sites in all known organisms cleave after the sequence EXXR (where X represents any amino acid residue; WO 00/48627 ; Uhlmann et al., 1999; Buonomo et al., 2000; Hauf et al., 2001) it is assumed that SFEILR^{1506} (SEQ ID NO:11) and EWELLR^{1535} (SEQ ID NO:12) represent two of the separase cleavage sites.

At page 30, the paragraph starting at line 26 and ending at page 31 line 18 was replaced with the following paragraph:

In order to establish a robust screening assay (based on liquid phase fluorescence energy transfer) for identifying inhibitors of recombinant human separase, four peptide substrates (1: SFEILR(SEQ ID NO:11)-AMC, 2: SFEILRG(SEQ ID NO:13)-AMC, 3:EWELLR(SEQ ID NO:12)-AMC and 4:

DREIMR(SEQ ID NO:9)-AMC) were synthesized. These peptides are linked to AMC (7-Amido-4-methylcoumarin), a fluorogenic group, which has been described for proteolytic assays, such as for trypsin (Zimmerman et al., 1977) and cathepsin B (Barrett and Kirschke, 1981). AMC serves as a donor fluorophore and in the case of the separase-specific peptide substrates the amino acid bonds of the peptides function as acceptor chromophores (Fig. 9A). The peptide substrates are cleaved at the P1′-AMC junction; by processing the peptide-AMC bond the unquenched AMC is set free and can be monitored as increasing fluorescence. The designed peptide substrates represent the intramolecular cis-cleavage site in separase itself (peptides 1-3) and the intermolecular trans-cleavage site in cohesin respectively (peptide 4). Since all these peptides contain an Arg at the P1′ site, the peptides could be easily tested by utilizing trypsin (specific recognition site at P1′: Arg or Lys). Except for peptide 2, which contains an additional Gly between P1′ (Arg) and the AMC residue, all peptide substrates could be efficiently cleaved (Fig. 9B) by trypsin as follows: Trypsin solution (Gibco 043-90317 FU) was diluted 1:1000 in Hepes buffer containing 20mM Herpes (pH: 7.7), 100mM KCl, 1mM $MgCl_2$, 0.1mM $CaCl_2$ and 1mM DTT (freshly added). 1 µl of peptide 1 (4mg/ml in DMSO) was added, mixed and measured in a Hitachi f-2000 fluorescence spectrophotometer (Ex: 355 and Em: 460nm). A typical kinetics is shown in Fig. 9B.

The existing Sequence Listing for the above-identified application was canceled and replaced with the substitute Sequence Listing appended hereto, and inserted the same at the end of the application.

In the Claims:

Claims 1, 3 and 5-10 were rewritten as follows:

1 (Once amended) A method for identifying a compound that modulates [has the ability of modulating] sister chromatid separation by inhibiting the proteolytic activity of separase, characterized in that an active separase in the form of

a) one or more separase fragments [fragment(s)], optionally upon activation in the presence of securin, or

b) the full-length separase upon activation in the presence of securin,

is incubated in the presence of a separase substrate, with a test compound and that modulating effect of the test compound on the proteolytic activity of the active separase is determined.

3. (Once amended) The method of claim 1 [or 2], wherein the active separase [(fragment)] is activated in a mitotic cell extract in the presence of securin.

5. (Once amended) The method of claim 1, wherein the separase substrate is a peptide comprising [that carries] a fluorogenic group, which upon processing of the polypeptide results in a change in fluorescence and that change in fluorescence is correlated with the separase activity.

6. (Once amended) The method of claim 5, wherein the separase substrate is a peptide selected from peptides containing the sequence DREIMR (SEQ ID NO:9), SFEILR (SEQ ID NO:11) or EWELLR (SEQ ID NO:12).

7. (Once amended) A peptide selected from peptides containing the sequence DREIMR (SEQ ID NO:9), SFEILR (SEQ ID NO:11) or EWELLR (SEQ ID NO:12) or a derivative [derivate] thereof.

8. (Once amended) The peptide of claim 7 or a derivative [derivate] thereof for the treatment of cancer.

9. (Once amended) A pharmaceutical [Pharmaceutical] composition comprising [containing as the active ingredient,] the peptide [(derivative)] of claim 7.

10. (Once amended) An inhibitor of separase [which has been] identified by [in] the method of claim 1 for human therapy.